MECHANISMS OF SULFUR MUSTARD-INDUCED METABOLIC INJURY

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ABSTRACT

Studies on the mechanism of metabolic injury induced by sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) have demonstrated that exposure of human epidermal keratinocytes in culture to HD induces time- and dose-dependent NAD^+ depletion and inhibition of glucose metabolism (Martens, Biochem. Pharmacol., in press). Both occurred relatively early after alkylation, preceding the loss of membrane integrity that is indicative of metabolic cell death. The inhibition of glycolysis induced by HD was only partially correlated with the depletion of NAD^+ and, thus, was not simply a function of changes in the NAD^+ level. Rather, HD appeared to induce complex shifts in the pattern of glucose metabolism that paralleled both the timing and degree of injury. In line with these findings, recent experiments have shown that partial protection against HD-induced NAD^+ depletion by 1 mM niacinamide did not protect against the inhibition of glycolysis. In preliminary experiments examining the effect of HD-induced metabolic changes on the cellular energy state, dose-dependent depletion of ATP was seen at 24 hours after exposure, but not at 4 or 8 hours. As seen for glucose metabolism, 1 mM niacinamide did not prevent the loss of this high-energy intermediate (ATP). We conclude from these studies that the relationships among HD exposure, glucose metabolism, and intracellular NAD^+ and ATP are more complex than originally proposed (Papirmeister et al, Fund. Appl. Toxicol. 5:S134, 1985).

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INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) [1] is a potent alkylating agent, inducing cross-links and strand breaks in DNA, as well as covalent modification of proteins and other cellular components. One recent model of HD-induced injury [2] links DNA damage to blister formation via poly(ADP-ribose) polymerase (PADPRP) induced NAD^+ depletion and consequent disturbances in intracellular metabolism. Previous studies in this laboratory and others [1,3-6] on the biochemical mechanisms of the cutaneous injury induced by sulfur mustard have demonstrated that exposure of cultured human epidermal keratinocytes (HEK) to HD induces time- and dose-dependent cytotoxicity, NAD^+ depletion and inhibition of glucose metabolism. However, the inhibition of glycolysis induced by HD was only partially correlated with the depletion of NAD^+ and, thus, was not simply a function of changes in the NAD^+ level [6]. Rather, HD appeared to induce complex shifts in the pattern of glucose metabolism that depended on both the timing and degree of injury.

Niacinamide, a PADPRP inhibitor and NAD^+ precursor, has been shown to protect HEK from the cytotoxic, histopathologic and NAD^+-depleting effects of HD *in vitro* for up to 24 hours after exposure [1,3-5,7]. In addition, niacinamide has been shown to prevent the loss of NAD^+ in both fresh human skin [8] and in the *in vivo* hairless guinea pig [9] as well as the development of HD-induced pathology in the latter model [9]. In order to better define the role of NAD^+ in the mechanism of HD-induced metabolic injury and the role of the metabolic injury in the initiation of pathology, the effects of niacinamide on the HD-induced inhibition of glucose metabolism were examined in cultured HEK. The results of these studies are reported here.

MATERIALS & METHODS

Materials: Human epidermal keratinocytes (HEK), Keratinocyte Growth Medium (KGM), Trypsin-EDTA and Trypsin Neutralizing Solution were purchased from Clonetics Corp. (San Diego, CA). Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) was obtained from the Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD). Stock HD (4 mM) was suspended in KGM; all further dilutions were made using KGM. Enzymes and fine chemicals were purchased from Sigma Chemical Co. All other chemicals were reagent grade or better and purchased from commercial suppliers.

HD Exposure and Niacinamide Treatment: HEK purchased as 2nd passage cells were maintained in KGM at 37°C under 5% CO_2 and subcultured as previously described [3]. Third to 5th passage cells were plated at 50-100,000 HEK/well in 24-well Falcon tissue culture plates (Becton-Dickinson & Co., Lincoln Park, NJ) in 1 mL of KGM. Upon reaching 80-100% confluence (100-150,000 HEK per well), the KGM was replaced with fresh KGM supplemented with 0, 0.01, 0.1 or 1.0 mM niacinamide. HD in KGM was added immediately after niacinamide to a final concentration of 0, 100 or 500 μM. Each condition was carried out in duplicate wells. The plates were allowed to stand 1 hour at room temperature in a fume hood for venting of

HD, after which they were returned to the incubator.

Cell counts and viabilities were determined on HEK harvested from both control and exposed/treated wells with trypsin-EDTA [3]. Cells were counted manually using a hemacytometer. Viabilities were determined by propidium iodide dye exclusion using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) [3].

Extractions: Control and exposed cells + medium were acid extracted with 0.5 M $HClO_4$ immediately upon addition of the fresh medium (t = 0 hr) and at subsequent intervals up to 24 hr. The samples were allowed to extract overnight at 4° and neutralized with 2 N KOH in 0.66 M phosphate buffer, pH 7.5 [10]. $KClO_4$(ppt) was removed by centrifugation and the supernatants stored frozen until assay. Alternatively, the cells + medium were solubilized with detergent (Somatic Cell ATP Releasing Reagent, Sigma Chemical Co., St. Louis, MO) and stored frozen for assay of ATP.

Assays: The NAD^+ contents of the extracted HEK were assayed in a final volume of 1.1 mL using the enzymatic cycling assay of Jacobson & Jacobson [10]. The buffer pH was increased from 7.8 to 8.5 to improve linearity. The coenzyme contents were calculated from standard curves constructed using NAD^+ solutions of known concentration determined by enzymatic endpoint assays [11].

Glucose and lactate levels in the extracts were assayed directly using a YSI Model 2700 dual analyzer (Yellow Springs Instruments, Yellow Springs, OH) fitted with glucose- and lactate-selective enzyme membranes. The rates of glucose metabolism in control and exposed wells were measured as the disappearance of glucose and the appearance of lactate as a function of time, and were calculated using least square regression analysis of the data.

ATP was assayed using the Sigma ATP bioluminescent assay kit according to the instructions provided (Sigma Technical Bulletin #BSCA-1, Sigma Chemical Co., St. Louis, MO, 1987). Bioluminescence was measured in an LKB-Wallac Model 1250 luminometer (LKB Instruments). The signal was calibrated with known concentrations of ATP determined from enzymatic endpoint assays coupled to the production of NADPH [12].

Student's t-test was used to assess statistical significance.

RESULTS & DISCUSSION

Cell Viability: Figure 1 shows the effects of niacinamide on the 24-hour viability of HEK exposed to HD. As can be seen, 100 μM HD in the absence or presence of added niacinamide had no effect on the membrane integrity of the cells as measured by dye exclusion. In contrast, 500 μM HD caused a 34% loss of viability at 24 hours. A slight though not significant protection (approx. 15%) from this loss was attained by the addition of either 0.1 or 1.0 mM niacinamide to the medium. This change was not as extensive as that seen previously in HEK [5]. In that study, niacinamide completely protected the viability at 24 hours under similar experimental conditions.

NAD^+ Content: At 4 hours after exposure, the intracellular content of NAD^+ was decreased to 46% of control by 500 μM HD, whereas 100 μM had no effect (Figure 2). Treatment of the cultures with increasing doses of niacinamide resulted in increasing protection against this depletion. At

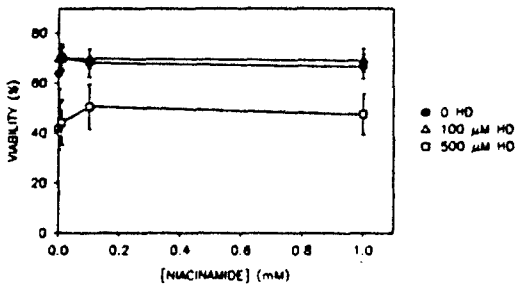

Figure 1. Effects of HD ± niacinamide on 24-hour viability as measured by dye exclusion. Experimental conditions as described in Materials & Methods. Data expressed as Mean ± SEM of 6 experiments.

1 mM added niacinamide, NAD^+ levels were maintained at 80% of those seen in the unexposed, untreated control, a significant level of protection ($P < 0.02$) when compared to 500 μM HD in the absence of niacinamide. Niacinamide did not significantly increase the levels of NAD^+ in either unexposed HEK or those exposed to 100 μM HD.

Dose-dependent NAD^+ depletion was seen at 24 hours after exposure to both 100 μM (63% of control) and 500 μM HD (27% of control), as can be seen in the left-hand panel of Figure 2. At this time point, significant protection of the NAD^+ levels against exposure to 100 μM HD was seen with 1 mM niacinamide ($P < 0.02$) when compared to 100 μM HD without niacinamide. This concentration of niacinamide maintained intracellular NAD^+ at 100% of the unexposed, untreated control. In contrast to the 4-

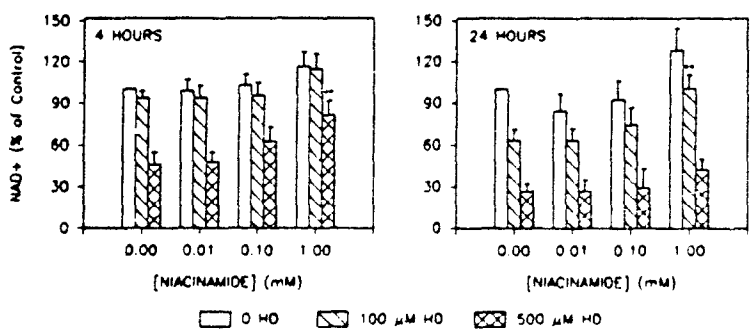

Figure 2. Effects of HD ± niacinamide on NAD^+ levels at 4 and 24 hours post-exposure. Experimental conditions as in Materials & Methods. Data expressed as Mean ± SEM of 8 (4 hr) or 7 (24 hr) experiments. Controls: 299 ± 47 pmoles/10^5 HEK (4 hr); 466 ± 88 pmoles/10^5 HEK (24 hr). **Significantly different from HD minus niacinamide ($P < 0.02$).

hour results, 1 mM niacinamide did not significantly protect NAD^+ against exposure to 500 μM HD. The apparent increase in the NAD^+ content of unexposed HEK (129% of control) at 1 mM niacinamide was not statistically significant ($P > 0.1$).

These results are consistent with earlier studies [3-5] showing significant protection of the intracellular NAD^+ content in HEK by 1 mM niacinamide. In addition, as was also seen in the previous studies, the level of protection afforded by the niacinamide pretreatment lessened as the injury increased in severity. That is, less protection was seen at higher doses of HD and longer times after exposure.

Glucose Metabolism: The effects of HD and 1 mM niacinamide on the rates of glucose metabolism by cultured HEK are shown in Figure 3. The the overall disappearance of glucose and the production of the glycolytic end-product, lactate were measured. As predicted by the results of our earlier studies [6], HD caused a dose-dependent inhibition of both activities, as evidenced by decreases in the slopes of the curves to approximately 75-80% of control and 35-40% of control for 100 μM and 500 μM HD, respectively. The rates calculated from these curves are given in Table 1. As can be seen, addition of up to 1 mM niacinamide provided no protection against the inhibition of glucose metabolism in these experiments. Data at 0.01 and 0.1 mM niacinamide (not shown) were similar to those at 1 mM.

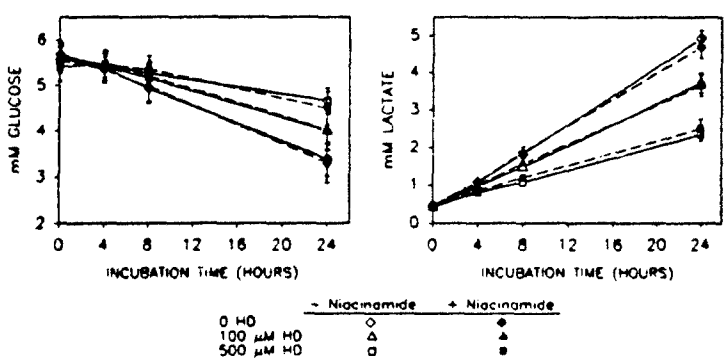

Figure 3. Effects of HD ± niacinamide on glucose utilization & lactate production. Experimental conditions as in Materials & Methods. [Niacinamide] = 1 mM. Data are Mean ± SEM of 5 (glu) or 7 (lac) experiments.

The inability of niacinamide to prevent the HD-induced inhibition of glucose metabolism in cultured HEK even under conditions in which NAD^+ levels were fully protected is in line with our earlier studies [6] which showed only a partial correlation between NAD^+ depletion and the inhibition of glycolysis. We concluded from those studies that the metabolic inhibition in HEK was not solely a function of NAD^+ depletion, but rather resulted from a combination of factors influenced by HD. That conclusion

is supported by the current results, as well as the similar findings by Mol et al [4] for glucose uptake by HEK. Interestingly, in later experiments on human skin cultures, Mol et al [8] observed protection against both NAD^+ depletion and inhibition of glucose uptake, but not the histopathology, suggesting differences in the *ex vivo* system that may need to be examined further.

Table 1

[HD] (μM)	[Niacinamide] (mM)	Glucose (μM/hour)	Lactate (μM/hour)
0	0	77 ± 20	189 ± 4
0	1.0	86 ± 23	177 ± 2
100	0	64 ± 22	138 ± 2
100	1.0	56 ± 25	133 ± 2
500	0	28 ± 26	79 ± 2
500	1.0	38 ± 22	85 ± 3

Effects of HD and niacinamide on the rates of glucose metabolism by HEK. Experimental conditions as described in Fig. 3.

ATP Content: As can be seen in Figure 4, incubation of HEK for 24 hours with 100 μM and 500 μM HD caused severe dose-dependent depletion of intracellular ATP (to 31% and 8% of control, respectively). As was seen for glucose metabolism, 1 mM niacinamide did not prevent the loss of this

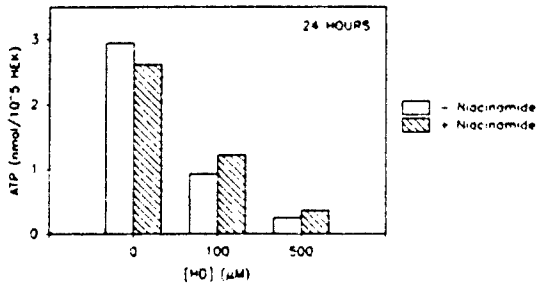

Figure 4. Effects of HD ± niacinamide on ATP levels at 24 hrs post-exposure. Experimental conditions are as in Materials & Methods. [Niacinamide] = 1 mM. Data are the mean of duplicate wells from 1 experiment.

high-energy intermediate. No depletion of ATP was apparent at 4 or 8 hours after exposure (not shown). Thus, as was seen for the inhibition of glucose metabolism, not only did the HD-induced depletion of ATP not parallel the loss of NAD^+, but it also could not be prevented by

niacinamide.

The effects on ATP reported here are consistent with those recently reported by by Mol et al [13]. However, both reports contrast with those of Meier et al [14], who reported ATP depletion in human resting lymphocytes as early as 30 minutes after exposure to 300 μM HD, as well as protection of the ATP levels by 1 mM niacinamide. However, lymphocytes have been shown [1,5] not only to be more sensitive to HD than HEK, but also, when exposed to HD, to exhibit to a somewhat different response to niacinamide as compared to HEK. Thus, the effects of HD and niacinamide on cellular energy metabolism also appear to be model-dependent.

In this paper, we have demonstrated that the protective effects of niacinimide in cultured HEK against HD-induced cytotoxicity and NAD^+ depletion do not extend to protection against the inhibition of glucose metabolism or depletion of the intracellular energy stores. These results, as well as those of others [1,4,13] support our original conclusion [6] that NAD^+ is not the sole cause of the metabolic inhibition that results from exposure to HD. Furthermore, this disconnect between NAD^+ depletion and metabolic inhibition may have implications for the use of niacinamide as an antidote/prophylactic for the treatment of HD-induced injuries.

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